

SHI et al., S.N. 10/526,682
Page 2

RECEIVED
APR 03 2009
CENTRAL FAX CENTER

Amendments to the Specification

The paragraph numbers specified below refer to the patent application published as US 2006/0127389 A1:

Please amend paragraph [0011], in the following manner:

[0011] In another aspect, the invention relates to a method for preparation of a fusion protein comprising a thrombolytic protein and an anticoagulant protein, which method comprises linking a thrombolytic protein gene and an anticoagulant protein gene via a base sequence encoding IEGR or [[GPR]] LGPR to form a gene encoding said fusion protein, and expressing said gene encoding the fusion protein in E.coli, yeast or animal cell lines to produce the fusion protein.

Please amend paragraph [0021], in the following manner:

[0021] As used herein, the term "linker peptide recognized by blood coagulation factor" refers to the tetrapeptide of IEGR (IleGluGlyArg), peptide containing IEGR sequence, ~~tripeptide~~ tetrapeptide of [[GPR]] LGPL (~~GlyProArg~~) LeuGlyProArg or peptide containing [[GPR]] LGPR sequence. As used herein, the term "diseases or conditions associated with thrombosis" refers to any disease or condition caused by thrombus, such as cerebral thrombus, arterial thrombus, stroke and atherosclerosis.

SHI et al., S.N. 10/526,682
Page 3

Dkt. 440/74021

Please amend paragraph [0024], in the following manner:

[0024] As used herein, the term "patient" refers to mammals, particularly human being. According to this invention, the fusion proteins of the invention is preferably a SFH fusion protein(SAK-GSIEGR-HV2) composed of staphylokinase and hirudin linked by GSIEGR, a fusion protein(tPA-PRIEGR-HV2) composed of tissue-type plasminogen activator(t-PA) and hirudin linked by PRIEGR, or a fusion protein (~~SAK-GSGPR-HV2~~) SAK-GSLGPR-HV2 composed of staphylokinase and hirudin linked by GSGPR GSLGPR.

Please amend paragraph [0028], in the following manner:

[0028] The thrombolytic activity of the purified fusion protein was determined using chromogenic substrate S-2251. To test thrombolytic and anticoagulant activity of the fusion protein *in vivo*, mouse-tail thrombosis [[[RTT)]]] MRTI was induced by kappa-carrageenin. The results show that the anticoagulant activity of the SFH fusion protein is significantly higher than that of SAK. In particular, after induction by kappa-carrageenin for 24 hrs, SAK is i.p. injected at a dose of 1.2mg/kg body weight every eight hours, and the inhibition of the tail thrombus is 36.6%. However, when equimolar SFH is administrated at a dose of 1.8mg/kg body weight, the inhibition of the tail thrombus is 100%. After induction by kappa-carrageenin for 36 hrs, the inhibition of the tail thrombus reaches 18.2% and 90% respectively by SAK and SFH administrated as above. The detailed results are shown in Tables 1-3.

SHI et al., S.N. 10/526,682

Dkt. 440/74021

Page 4

Please amend paragraph [0030] in the following manner.

TABLE 3

In vivo ~~anticoagulation~~ anti-thrombus activities of the fusion protein SFH and staphylokinase (SAK) (n= 10)

kappa-carrageenin-inducing time (hrs)	No. animals in each group	SAK	SFH
24	10	36.6%	100%
36	10	18.2%	90%

Note:

SAK is i.p. administrated at a dose of 1.2 mg/kg body weight every eight hours, and equimolar SFH is administrated at a dose of 1.8 mg/kg. The animals used are Kunming mice (KM mice). ~~The anticoagulant activity is expressed as the inhibition of mouse tail thrombosis.~~

Please amend paragraph [0031] in the following manner.

[0031] Table 1 shows that the fusion protein SFH exhibits the same level of thrombolytic activity as free staphylokinase. Table 2 shows that the intact fusion protein SFH does not exhibit anticoagulant activity, but shows entire ~~anticoagulant~~ anti-thrombus activity once cleaved by the blood coagulant factor FXa. Table 3 demonstrates that SFH has the significant anticoagulant effect. Thus the fusion protein of the invention indeed

SHI et al., S.N. 10/526,682
Page 5

Dkt. 440/74021

has both the thrombolytic and anticoagulant activities, and has lower side effect of hemorrhage.

Please amend paragraph [0032] in the following manner.

[0032] *Xho* I and *Avr* II restriction sites are added to the upstream and downstream of tPA gene, respectively. The tPA gene without the stop codon is introduced in the vector pPIC9. The *Avr* II restriction site and the sequence coding FXa recognition sequence are incorporated upstream of hirudin gene via a primer using PCR method. The primer matched with downstream of the hirudin gene contains a *Not* I restriction site. The hirudin gene with a FXa recognition sequence is digested by two enzymes of *Avr* II and *Not* I, and the resulting fragment is linked into the above constructed vector pPIC9, wherein the introduced fragment is located downstream of tPA gene and forms ~~together with tPA~~ into the fusion gene ~~[[PAFH]]~~ TFH. Thus constructed plasmid is designed as ~~pPAFH~~ pPIC9-TFH. The plasmids ~~pPAFH~~ pPIC9-TFH and pPIC9K are digested by *Bam*H I and *Sal* I. The ~~[[PAFH]]~~ TFH gene is then inserted into pPIC9K to form pPIC9K-TFH gene. The plasmid ~~pPAFH-K~~ pPIC9K-TFH is linearized, and incorporated into yeast genome by electrotransformation. Methanol is used to induce the expression. The desired fusion protein TFH comprises three domains, a tPA sequence, FXa recognition sequence and hirudin.

SHI et al., S.N. 10/526,682
Page 6

Dkt. 440/74021

Please amend paragraph [0033] in the following manner.

[0033] *EcoR* I and BamH I restriction sites are added to the two ends of SAK gene, respectively. The SAK gene without the stop codon is introduced in the vector pBV220, resulting in pBVSAK. The BamH I restriction site and the sequence coding [[FXIIa]] FIIa recognition sequence GSLGPR are incorporated upstream of hirudin gene via a primer using PCR method. The primer matched with downstream of the hirudin gene contains a Pst I restriction site. The hirudin gene with a [[FXIIa]] FIIa recognition sequence GSLGPR is digested by two enzymes of BamH I and Pst I, and the above vector pBVSAK is also digested by BamH I and Pst I. The digested hirudin fragment is inserted into the digested vector pBVSAK to form plasmid pBVSTH. The sequence is confirmed by enzymatic digestion. Alternatively, said two gene fragments may be linked by overlapping PCR method. The plasmid pBVSTH is transformed into *E.coli*, and induced to express at 42 °C. The desired fusion protein (STH) is obtained by ion exchange and gel filtration method in a purity of more than 96%. The STH fusion protein" comprises three domains, a SAK sequence, [[FXIIa]] FIIa recognition sequence GSLGPR and hirudin.